

## Enzymatic synthesis of biotin-labeled polynucleotides: Novel nucleic acid affinity probes

(nucleotide analog/DNA and RNA polymerase/avidin-Sepharose/antibiotin antibody/immunoprecipitation)

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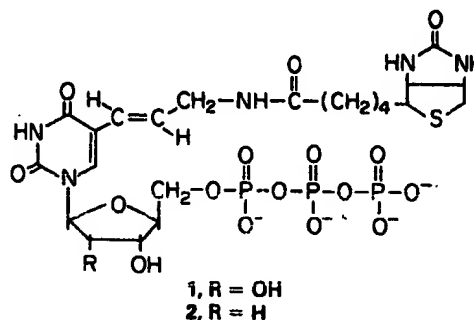
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**ABSTRACT** Analogs of dUTP and UTP that contain a biotin molecule covalently bound to the C-5 position of the pyrimidine ring through an allylamine linker arm have been synthesized. These biotin-labeled nucleotides are efficient substrates for a variety of DNA and RNA polymerases *in vitro*. Polynucleotides containing low levels of biotin substitution (50 molecules or fewer per kilobase) have denaturation, reassociation, and hybridization characteristics similar to those of unsubstituted controls. Biotin-labeled polynucleotides, both single and double stranded, are selectively and quantitatively retained on avidin-Sepharose, even after extensive washing with 8 M urea, 6 M guanidine hydrochloride, or 99% formamide. In addition, biotin-labeled polynucleotides can be selectively immunoprecipitated in the presence of antibiotin antibody and *Staphylococcus aureus* protein A. The unique features of biotin-labeled polynucleotides suggest that they will be useful affinity probes for the detection and isolation of specific DNA and RNA sequences.

Nucleotide analogs that can function as indicator "probes" when incorporated in polynucleotides would be of significant utility in many procedures used in biomedical and recombinant DNA research. When used in conjunction with immunological, histochemical, or affinity detector systems, such reagents could provide suitable alternatives to radioisotopes for the detection, localization, and isolation of nucleic acid components. Biotin (vitamin H) has many features that make it an ideal probe candidate. The interaction between biotin and avidin, a 68,000-dalton glycoprotein from egg white, has one of the highest binding constants ( $K_{d11} = 10^{-15}$ ) known (1). When avidin is coupled to appropriate indicator molecules (fluorescent dyes, electron-dense proteins, enzymes, or antibodies), minute quantities of biotin can be detected (2-8). The specificity and tenacity of the biotin-avidin complex has been exploited to develop methods for the visual localization of specific proteins, lipids, and carbohydrates on or within cells (for review, see ref. 2). Davidson and associates (9-11) chemically crosslinked biotin to RNA, via cytochrome *c* or polyamine bridges, and used these RNA-biotin complexes as probes for *in situ* hybridization. The sites of hybridization were visualized in the electron microscope through the binding of avidin-ferritin or avidin-methacrylate spheres. Although this approach to the detection of polynucleotide sequences was successful in the specialized cases examined, a simpler and more general procedure for preparing biotin-substituted nucleic acids was desirable. Biotin directly attached to a nucleotide that functions as an efficient polymerase substrate would be more versatile, both in the experimental protocols and in the detection methods that could be used.

We have synthesized a number of nucleotide analogs that contain potential probe determinants (e.g., biotin, iminobiotin,

and 2,4-dinitrophenyl groups) covalently attached to the pyrimidine or purine ring in the hope that one of them might prove to be a useful affinity reagent. This report describes the synthesis of biotin-labeled derivatives of UTP and dUTP (1 and 2, respectively) that are substrates for RNA or DNA polymerases. The properties of the resulting biotin-substituted polynucleotides appear to satisfy the basic criteria required of a good affinity probe.



### MATERIALS AND METHODS

**Materials.** Standard NTPs were purchased from P-L Biochemicals, and dUTP was obtained from Sigma. Radiolabeled nucleotides were products of New England Nuclear or Amersham Radiochemicals. *Escherichia coli* DNA polymerase I, both holoenzyme and Klenow fragment, was obtained from Boehringer Mannheim; restriction enzymes were from New England Biolabs or Bethesda Research Laboratories. The following enzymes and reagents were gifts: T7 RNA polymerase and T7 DNA (J. Coleman); herpes simplex DNA polymerase (B. Francke); L1210 and HeLa cell DNA polymerases  $\alpha$  and  $\beta$  (H. S. Allaudeen); avian myeloblastosis reverse transcriptase (RNA-dependent DNA nucleotidyltransferase) (S. Weissman); murine and calf thymus RNA polymerase II (R. Roeder); *E. coli* RNA polymerase (P. Farnam); and rabbit antibiotin serum (F. Harman).

**Synthesis of 1 (Bio-UTP) and 2 (Bio-dUTP).** *Mercuriation step.* The 5-mercurated derivatives of UTP and dUTP were prepared by a modification of the procedure of Dale *et al.* (12). UTP (570 mg, 1.0 mmol) or dUTP (554 mg, 1.0 mmol) in 100 ml of 0.1 M sodium acetate, pH 6.0, was treated with mercuric acetate (1.59 g, 5.0 mmol). The solution was heated at 50°C for 4 hr and then cooled on ice. Lithium chloride (392 mg, 9.0 mmol)

Abbreviations: AA-UTP and AA-dUTP, 5-(3-amino)allyl uridine and deoxyuridine triphosphates, respectively; Bio-UTP and Bio-dUTP, 5-allylaminobiotin-labeled UTP and dUTP, respectively; Bio-RNA and Bio-DNA, biotin-labeled RNA and DNA, respectively; MVM, minute virus of mouse; RF, replicative form.

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was added, and the solution was extracted six times with equal volumes of ethyl acetate to remove excess  $\text{HgCl}_2$ . The efficiency of the extraction process was monitored by estimating the mercuric ion concentration in the organic layer by using 4,4'-bis(dimethylamino)-thiobenzophenone (13). The extent of nucleotide mercuriation, determined spectrophotometrically by following the iodination of an aliquot of the aqueous solution (14), was routinely 90–100%. The nucleotide products in the aqueous layer, which often became cloudy during the ethyl acetate extraction, were precipitated by the addition of 3 vol of ice-cold ethanol and collected by centrifugation. The precipitate was washed twice with cold absolute ethanol and once with ethyl ether and then air dried. These products were used for the synthesis of the 5-(3-amino)allyl nucleotides without further purification.

**Synthesis of 5-(3-amino)allyluridine and deoxyuridine 5' triphosphates (AA-UTP and AA-dUTP).** Organomercurial compounds can be alkylated or arylated under mild conditions by reaction with olefins in the presence of a palladium catalyst (15). Bergstrom and associates (16, 17) have recently used this procedure for the synthesis of C-5-substituted pyrimidine nucleosides. We have also adopted this general synthetic approach for introducing the allylamine linker arm. The mercurated nucleotides were dissolved in 0.1 M sodium acetate, pH 5.0, and adjusted to 20 mM ( $A_{287}$ , 200 units/ml). A fresh 2.0 M solution of allylamine (Aldrich) was prepared by slowly adding 1.5 ml of allylamine (13.3 M) to 8.5 ml of ice-cold 4 M acetic acid. Three milliliters (6.0 mmol) of the neutralized allylamine stock was added to 25 ml (0.5 mmol) of nucleotide solution. One nucleotide equivalent of  $\text{K}_2\text{PdCl}_4$  (163 mg, 0.5 mmol; Alfa-Ventron, Danvers, MA) in 4 ml of water was then added to initiate the reaction; the solution gradually turned black and metal (Hg and Pd) deposits appeared on the walls of the reaction vessel. After standing at room temperature for 18–24 hr, the reaction mixture was passed through a 0.45- $\mu\text{m}$  membrane filter (Nalgene) to remove most of the remaining metal precipitate. The yellow filtrate was diluted 1:5 with  $\text{H}_2\text{O}$  and applied to a 100-ml column of DEAE-Sephadex A-25 (Pharmacia). After washing with 1 column vol of 0.1 M sodium acetate, pH 5.0, the products were eluted by using a 1-liter linear gradient (0.1–0.6 M) of sodium acetate, pH 8–9, or  $\text{Et}_3\text{NHCO}_3$ , pH 7.5. The desired product was in the major UV-absorbing peak, which eluted between 0.30 and 0.35 M salt. Because spectral analysis showed that this peak contained several products, final purification was achieved by reverse-phase high-pressure liquid chromatography on columns of Partisil-ODS2, using either 0.5 M  $(\text{NH}_4)_3\text{PO}_4$ , pH 3.3 (analytical separations), or 0.5 M  $\text{Et}_3\text{NHOAc}$ , pH 4.3 (preparative separations), as eluents. AA-UTP and AA-dUTP were the last peaks to elute from the column and they were cleanly resolved from three as-yet unidentified contaminants. The characterization of the (3-amino)allyl nucleotides by proton NMR, elemental, spectral, and chromatographic analyses will be presented in detail elsewhere. These studies clearly showed that the (3-amino)allyl substituent is attached to the C-5 position of the pyrimidine ring and that the olefinic protons are in the *trans* configuration.

**Conversion of AA-UTP or AA-dUTP to Bio-UTP and Bio-dUTP.** Biotinyl-*N*-hydroxysuccinimide ester was prepared from biotin (Sigma) as described (3). AA-UTP $\cdot 4\text{H}_2\text{O}$  (70 mg, 0.1 mmol) or AA-dUTP $\cdot \text{H}_2\text{C}$  (63 mg, 0.1 mmol) in 20 ml of 0.1 M sodium borate, pH 8.5, was treated with the ester (34.1 mg, 0.1 mmol) in 2 ml of dimethylformamide. The reaction mixture was left at room temperature for 4 hr and then loaded directly onto a 30-ml column of DEAE-Sephadex A-25 previously equilibrated with 0.1 M  $\text{Et}_3\text{NHCO}_3$ , pH 7.5. The column was eluted with a 400-ml linear gradient (0.1–0.9 M) of  $\text{Et}_3\text{NHCO}_3$ . Frac-

tions containing bio-dUTP or bio-UTP, which eluted at 0.55–0.65 M  $\text{Et}_3\text{NHCO}_3$ , were desalted by rotary evaporation in the presence of methanol and then dissolved in water. Occasionally, a slightly cloudy solution was obtained; this turbidity, due to a contaminant in some  $\text{Et}_3\text{NHCO}_3$  solutions, was removed by filtration through a 0.45- $\mu\text{m}$  filter. For long-term storage, the nucleotides were converted to sodium salts by briefly stirring the solution in the presence of Dowex 50 ( $\text{Na}^+$ ). After filtration, the nucleotide was precipitated by the addition of 3 vol of cold ethanol, washed with ethyl ether, dried at reduced pressure over sodium hydroxide pellets, and stored in a desiccator at  $-20^\circ\text{C}$ . For immediate use, the nucleotide solution was made 20 mM in Tris-HCl, pH 7.5, and adjusted to a final nucleotide concentration of 5 mM. Stock solutions were stored at  $-20^\circ\text{C}$ .

**Analysis.** Bio-dUTP: Calcd. for  $\text{C}_{22}\text{H}_{30}\text{N}_5\text{O}_{16}\text{P}_3\text{SNa}_4\cdot\text{H}_2\text{O}$ : C, 29.80; H, 3.38; N, 7.89; P, 10.47; S, 3.61. Found: C, 30.14; H, 3.22; N, 7.63; P, 10.31; S, 3.70. Bio-UTP: Calcd. for  $\text{C}_{22}\text{H}_{30}\text{N}_5\text{O}_{16}\text{P}_3\text{SNa}_4\cdot 3\text{H}_2\text{O}$ : C, 29.15; H, 3.19; N, 7.45; P, 9.89; S, 3.41. Found: C, 28.76; H, 3.35; N, 7.68; P, 9.81; S, 3.32. The spectral properties of bio-dUTP and bio-UTP at pH 7.5 [ $\lambda_{\text{max}}$  289 nm ( $\epsilon = 7100$ );  $\lambda_{\text{max}}$  240 nm ( $\epsilon = 10,700$ );  $\lambda_{\text{min}}$  262 nm ( $\epsilon = 4300$ )] reflect the presence of an exocyclic double bond conjugated with the pyrimidine ring. These nucleotides also give a strong positive reaction (an orange-red color) when treated with *p*-dimethylaminocinnamaldehyde in ethanolic sulfuric acid, a procedure used for biotin quantitation (18). However, in contrast to AA-dUTP and AA-UTP, they do not give a positive ninhydrin reaction.

## RESULTS

Bio-UTP and Bio-dUTP have been synthesized. These analogs were then tested for their ability to function as substrates for a series of purified nucleic acid polymerases *in vitro*. As shown in Fig. 1, Bio-dUTP is an excellent substrate for *E. coli* DNA polymerase I using either the nick-translation protocol of Rigby *et al.* (19) or the "gap-filling" reaction described by Bourguignon *et al.* (21). Although it is incorporated at an initial rate that is only 30–40% of that of the control reaction with TTP, the final specific activities (and the extent of polymerization) that can be achieved are essentially the same. Bio-dUTP is also a substrate for bacteriophage T4 DNA polymerase, DNA polymerases  $\alpha$  and  $\beta$  from murine (A-9) and human (HeLa) cells, and the DNA polymerase of herpes simplex virus, with incorporation efficiencies similar to that of *E. coli* DNA polymerase I (not shown). In addition, Bio-dUTP will support DNA synthesis in a nuclear replication system prepared from baby hamster kidney cells infected with herpes simplex virus (unpublished data). In contrast, Bio-dUTP does not function as a substrate for avian myeloblastosis virus reverse transcriptase under standard incubation conditions using mRNA-oligo(dT), minute virus of mouse (MVM) DNA, or poly(dA)-oligo(dT) as template-primer complexes.

The ribonucleotide analog, Bio-UTP, can substitute for UTP in reactions catalyzed by the RNA polymerases of *E. coli* and bacteriophage T7 (Fig. 2), although with a lower efficiency than that of any DNA polymerase/Bio-dUTP system. Furthermore, Bio-UTP is utilized poorly, if at all, by the eukaryotic RNA polymerases we have examined (HeLa cell RNA polymerase III, calf thymus RNA polymerase II, and mouse L-cell RNA polymerase II). Although the limited range of substrate function precludes the use of Bio-UTP in the direct enzymatic biotin labeling of eukaryotic transcripts *in vivo*, biotin-labeled RNA (Bio-RNA) probes can be prepared *in vitro* by using appropriate DNA templates and *E. coli* RNA polymerase or by 3'-end labeling methods using RNA ligase and biotin-labeled pUp (not shown).

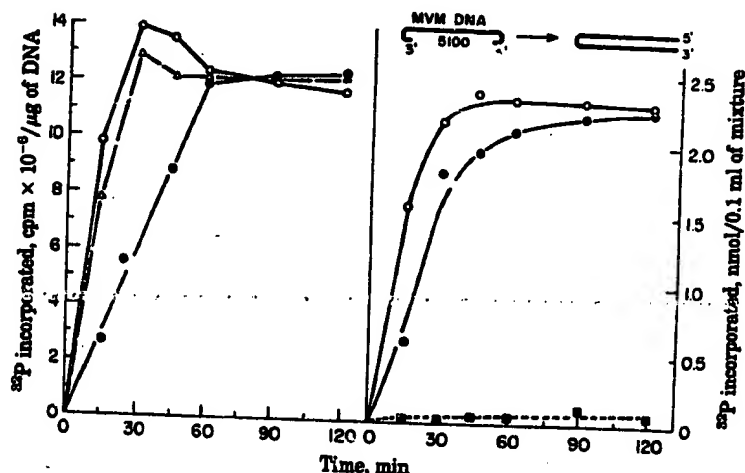


FIG. 1. Bio-dUTP is a substrate for *E. coli* DNA polymerase I. (A) A Phage DNA was nick translated *in vitro* with DNA polymerase I holo-enzyme as described (19). Reactions used [ $^{32}$ P]dATP (1.35  $\mu$ M, 400 Ci/ $\mu$ mol) and either 20  $\mu$ M TTP ( $\circ$ ), 20  $\mu$ M Bio-dUTP ( $\bullet$ ), or 10  $\mu$ M TTP/10  $\mu$ M Bio-dUTP ( $\Delta$ ). (B) Minute virus of mouse (MVM) DNA, a 5-kilobase single-stranded DNA with terminal hairpin duplexes (20), was converted to a double-stranded form by reaction with DNA polymerase I (Klenow fragment) as described (21). The three-nucleotide reaction ( $\square$ ; no TTP) contained dCTP, dGTP, and [ $^{32}$ P]dATP (50  $\mu$ Ci/ $\mu$ mol) at 0.1 mM. TTP ( $\circ$ ) and Bio-dUTP ( $\bullet$ ) reactions were supplemented with the appropriate triphosphate at a final concentration of 0.1 mM.

The enzymatic polymerization of nucleotides containing biotin was not monitored directly because neither Bio-dUTP or Bio-UTP were radiolabeled. However, two lines of evidence show that the biotin-labeled nucleotides are incorporated. The first is that polynucleotides synthesized in the presence of biotin-labeled nucleotides are selectively retained when chromatographed over avidin-Sepharose affinity columns. For example, normal DNA, nick translated with TTP, dCTP, dGTP, and [ $^{32}$ P]dAMP, is quantitatively eluted from avidin-Sepharose by the addition of 0.5 M NaCl. In contrast, the majority of nick-translated biotin-labeled DNA (Bio-DNA) remains bound to the resin even after extensive washing with high salt, urea, guanidine-HCl, formamide, 2 mM biotin, or 50 mM NaOH (Table 1). The small fraction of radiolabel eluted by these washing conditions is not retained when it is applied to the resin a second time, suggesting that this radioactivity is associated with DNA fragments that are free of biotin substitution. Because the pBR322 DNA used in this experiment had  $\approx 5\%$  of its thymidine residues substituted by Bio-dUMP (based on picomoles of [ $^{32}$ P]dAMP incorporated in the nick-translation reaction), it is clear that only a few molecules of biotin per kilobase of DNA are necessary for irreversible binding to avidin-Sepharose. Indeed, when the "sticky" ends of Simian virus 40 DNA (linearized by treatment with *Eco*RI) are filled in by using Bio-dUTP and *E. coli* DNA polymerase Klenow fragment, the DNA is retained on avidin-Sepharose (unpublished data). Thus, four biotin molecules or fewer per five kilobases of DNA are sufficient for selective retention.

The second line of evidence for biotin substitution is that only polynucleotides synthesized in the presence of biotin-labeled nucleotides are immunoprecipitated when treated with purified anti-biotin antibodies and then with formalin-fixed *Staphylococcus* (Table 2). Although the amount of biotin-labeled polymer found in the immune precipitate is dependent on the antibody concentration and time of incubation, under optimum conditions,  $>90\%$  of the product can be immunoprecipitated, even when present in subnanogram quantities. Significantly, the results in Tables 1 and 2 show that the biotin molecule can be recognized by avidin and anti-biotin antibodies when the DNA is still in a double-stranded form. Parallel experiments (not shown) indicate that biotin-labeled DNA-RNA hybrids and RNA duplexes behave similarly. These observations suggest that immunological and affinity methods could be used for probe detection (or isolation) following standard hybridization procedures.

To determine whether biotin-substituted polynucleotides

were suitable for use as hybridization probes, the denaturation and renaturation characteristics of several biotin-labeled DNA and RNA polymers were examined. As shown in Table 3, the melting temperature of DNA duplexes decreases as the Bio-dUMP content of the polymer increases. A parallel analysis of RNA duplexes and DNA-RNA hybrids (not shown) indicates that they respond similarly. However, a pronounced decrease in melting temperature occurs only in heavily substituted polymers [e.g., poly(dA-dBio-U)] and even then the degree of coop-

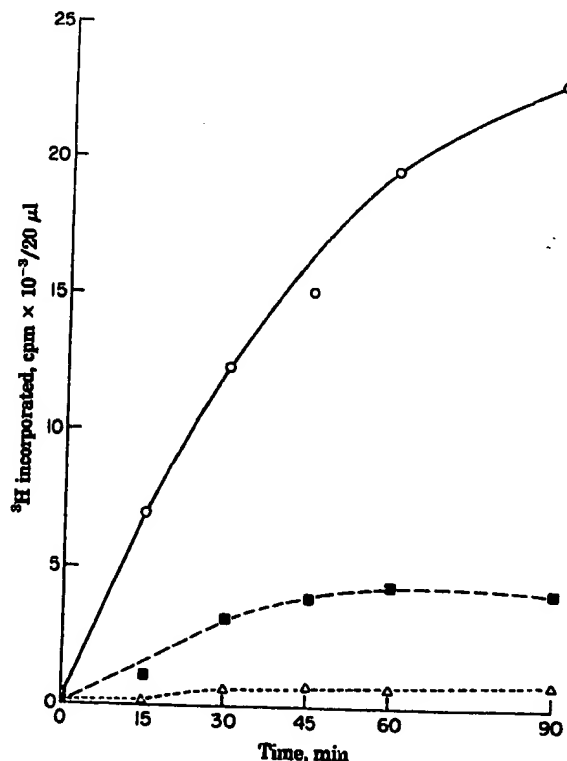


FIG. 2. Bio-UTP is a substrate for T7 RNA polymerase. Reaction mixtures (0.15 ml) were 40 mM Tris-HCl, pH 8.0/30 mM MgCl<sub>2</sub>/10 mM 2-mercaptoethanol/0.4 mM ATP/0.4 mM GTP/0.4 mM [ $^3$ H]CTP (100  $\mu$ Ci/ $\mu$ mol)/0.4 mM UTP or Bio-UTP containing 7.5  $\mu$ g of T7 DNA and 3.2  $\mu$ g of T7 RNA polymerase. Aliquots (20  $\mu$ l) were removed at the indicated times and acid precipitated onto glass-fiber filters. Curves:  $\circ$ , UTP;  $\bullet$ , Bio-UTP;  $\Delta$ , three-nucleotide reaction; no UTP or Bio-dUTP.

Table 1. Selective retention of biotin-labeled DNA on avidin-Sepharose

	% DNA retained on resin	
	Bio-DNA (5%)*	Control
Load	100	100
Eluent		
0.5 M NaCl	100	0.1
1.0 M NaCl	99.7	<0.01
8 M Urea	100	<0.01
6 M Guanidine-HCl	95.2	<0.01
99% Formamide	94.7	<0.01
2 mM Biotin	97.6	<0.01
50 mM NaOH	89.5	<0.01

Avidin-Sepharose was prepared by coupling avidin to cyanogen bromide-activated Sepharose 4B essentially as described (22). Columns containing 0.2 ml of resin were equilibrated with 10 mM Tris-HCl/0.20 M NaCl, pH 7.5, and the DNA samples ( $3 \times 10^6$  cpm per load;  $2 \times 10^7$  cpm/ $\mu$ g) were applied in 0.2 ml of the same buffer. The columns were washed with 1.0 ml of loading buffer and then treated with 2.0 ml of eluent. The % DNA retained on the resin was calculated by using the formula (cpm of DNA loaded - cpm eluted) + cpm loaded, as determined by Cerenkov counting.

\* pBR322 DNA labeled with [ $^{32}$ P]dAMP by nick translation in which 5% of the TMP residues have been replaced by Bio-dUMP.

erativity and the extent of hyperchromicity observed during denaturation are virtually identical to that of control polymers. Thus, pBR322 or  $\lambda$  DNAs that have been nick translated to introduce  $\approx 20$  biotin molecules per kilobase have melting temperatures similar to those of their biotin-free counterparts. Even MVM replicative form (RF) DNA in which every TMP residue in one strand ( $\approx 1250$  in 5 kilobases) is replaced by Bio-dUMP has a melting temperature that is only 5°C less than that of the unsubstituted DNA. Of greater significance is the observation that lightly labeled DNA probes hybridize in solution at essentially the same rate as biotin-free probes (Fig. 3). Furthermore,  $^{32}$ P-labeled biotin-substituted pBR322 DNA has the same degree of specificity and autoradiographic signal intensity as control biotin-free pBR322 DNA when used as a hybridization probe for detecting bacterial colonies that contain the plasmid (data not shown). These results indicate that a substantial number of biotin-labeled nucleotides can be introduced into a

Table 2. Selective immunoprecipitation of Bio-DNA with anti-biotin IgG and Staphylococcus

DNA	Antibody	Radioactivity, cpm	
		Precipitate	Supernatant
Control	—	70	4867
Control	Anti-Bio IgG	87	5197
Control	Nonimmune IgG	55	5107
Bio-DNA	—	53	3886
Bio-DNA	Anti-Bio IgG	3347	736
Bio-DNA	Nonimmune IgG	60	3900

Immunoprecipitation of DNA samples was done essentially as described (23). Biotin-labeled and control pBR322 DNAs labeled with [ $^{32}$ P]dAMP by nick translation (specific activity,  $2 \times 10^7$  cpm/ $\mu$ g) were treated with 100  $\mu$ l of formalin-fixed Staphylococcus (IgG Sorb, The Enzyme Center) in water for 10 min at room temperature. The supernatants from these reaction mixtures were incubated at 4°C for 1 hr without serum, with nonimmune rabbit serum, or with rabbit anti-biotin affinity purified from serum provided by Fred Harmon. Immune complexes were precipitated by the addition of 50  $\mu$ l of IgG Sorb. After 10 min at room temperature, the mixtures were centrifuged, and the pellets were washed three times with 30 mM Tris-HCl/150 mM NaCl/0.05% Nonidet P-40, pH 7.5, and analyzed by Cerenkov counting.

Table 3. Effect of biotin substitution on the thermal denaturation of DNA duplexes

DNA	Bio-dUMP content (% total nucleotides)	$T_m$ , °C
Control pBR322	—	80
Biotin-labeled pBR322	2.0	79
Control MVM RF	—	69
Biotin-labeled MVM RF	12.5	64
poly(dA-dT)	—	62
poly(dA-dBio-U)	50.0	47

pBR322 DNAs were prepared by nick translation and thermally denatured in 10 mM Tris-HCl/50 mM NaCl/1.0 mM EDTA, pH 7.5. MVM RF DNAs were prepared as described in the legend to Fig. 1, and melting profiles were determined in 10 mM Tris-HCl/1.0 mM EDTA, pH 7.5. poly(dA-dT) and poly(dA-dBio-U) were prepared from *E. coli* DNA polymerase I reactions primed by poly(dA-dT) as described (24), and melting profiles were determined in 10 mM Tris-HCl/0.10 M NaCl/1.0 mM EDTA, pH 7.5.

nucleic acid probe without significantly altering its hybridization characteristics.

Several additional properties of biotin-labeled polynucleotides are worth noting at this point. First, phenol extraction should be avoided whenever possible during purification of Bio-DNA or Bio-RNA because heavily substituted polymers are extracted into the phenol layer and even lightly or moderately substituted ones (e.g., nick-translated DNAs) can often be retained at the phenol/H<sub>2</sub>O interface. Second, because the mass of Bio-dUMP is about twice that of TMP, extensive substitution can appreciably increase the overall mass of the polymer. For example, biotin-labeled MVM RF DNA (Fig. 1B) and restriction fragments derived from it migrate more slowly in agarose gels than their biotin-free counterparts (Fig. 4). Finally, incorporation of a biotin-labeled nucleotide into a restriction endonuclease recognition site may prevent enzymatic cleavage.

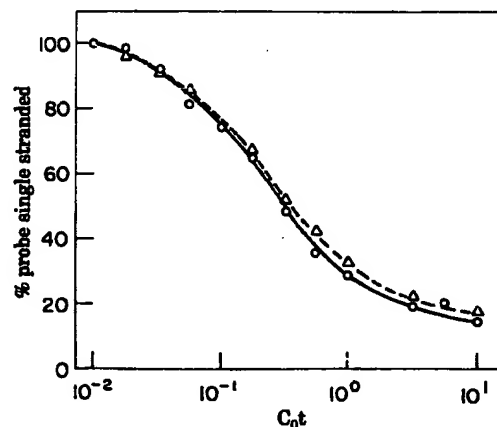


Fig. 3. Effect of biotin substitution on the reassociation rate of *E. coli* DNA. Sheared *E. coli* DNA was nick translated by using [ $\alpha$ - $^{32}$ P]dATP and either TTP or Bio-dUTP to a specific activity of  $1.3 \times 10^6$  cpm/ $\mu$ g; the Bio-DNA probe had  $\approx 5.5\%$  of its TMP residues replaced by Bio-dUMP. The probes were heat denatured and hybridized at 37°C to a 220-fold excess of denatured nonradiolabeled *E. coli* DNA in 50% formamide/0.30 M NaCl/0.03 M sodium citrate, pH 7.0. Aliquots (10  $\mu$ l) were removed at various times and diluted into 100  $\mu$ l of 0.05 M sodium acetate, pH 5.0/0.05 M NaCl/1.0 mM ZnCl<sub>2</sub>. Mung bean nuclease (2 units; P-L Biochemicals) was added, and the mixture was incubated at 42°C for 15 min. The amount of  $^{32}$ P-labeled probe made resistant to the single-strand-specific nuclease was determined by acid precipitation onto glass-fiber filters.  $C_0t$ , initial concentration of DNA (moles of nucleotide/liter)  $\times$  time (sec).  $\circ$ , Control DNA;  $\Delta$ , Bio-DNA probe.

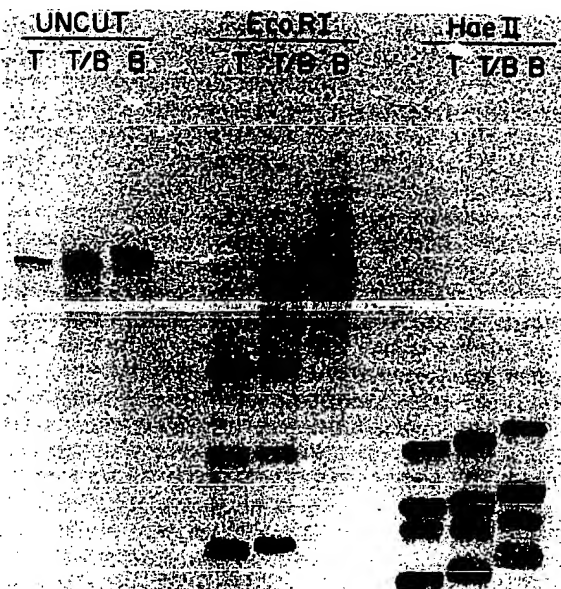


FIG. 4. Effect of biotin substitution on restriction endonuclease cleavage of DNA. <sup>32</sup>P-labeled MVM RF DNAs prepared as described in the legend to Fig. 1B were cleaved with *Eco*RI and *Hae* II as described (20), and the samples were subjected to electrophoresis in a 1.4% agarose gel. Lanes: T, DNA gap filled with TTP as a substrate; T/B, DNA gap filled in the presence of equimolar concentrations of TTP and Bio-dUTP; B, DNA gap filled with Bio-dUTP. The faint bands in the *Hae* II lanes reflect a trace contamination of the enzyme with *Hae* III.

When Bio-dUMP is in the recognition site of *Eco*RI (C-A-A-T-T-C), the DNA is totally refractory to this enzyme although it remains sensitive to digestion by *Hae* II (PuG-C-G-CPy) (Fig. 4).

# DISCUSSION

Our data demonstrate that Bio-dUTP and Bio-UTP are used as substrates by a number of nucleic acid polymerases, albeit at somewhat lower rates than the parent compounds, TTP and UTP. This provides a simple and rapid procedure for synthesizing chemically stable biotin-substituted polymers that hybridize specifically and efficiently to complementary sequences either in solution or bound to solid supports. Because polynucleotides containing a limited number of biotin molecules (50 or fewer per kb) hybridize with kinetics similar to those of unlabeled controls, standard hybridization protocols need be modified little if at all. The observation that Bio-DNA or Bio-RNA, and nonbiotinized sequences that hybridize to them, are selectively retained on avidin-Sepharose columns or immunoprecipitated by the addition of anti-biotin antibodies and Staphylococcus is significant in several regards. First, these results suggest that biotin-labeled polymers can be used in conjunction with appropriate immunofluorescent, immunohistochemical, or affinity reagents for detecting or localizing specific sequences in

chromosomes, cells, tissue sections, and blots. Our studies have led to the development of a rapid method of gene mapping by *in situ* hybridization that uses rabbit anti-biotin antibody and fluorescein-labeled goat anti-rabbit IgG to identify the loci of hybridized Bio-DNA probes and a histochemical procedure for detecting biotin-labeled sequences on nitrocellulose filters that uses antibody-alkaline phosphatase conjugates (unpublished data). Second, the ability to synthesize immunogenic DNAs (and to a lesser extent RNAs) enzymatically, both in purified *in vitro* systems and in crude cell lysates, may allow the use of immunoprecipitation techniques. Finally, because the interaction between biotin-labeled polynucleotide probes and avidin-Sepharose is essentially irreversible, it should be possible to develop refined protocols for enriching (or deleting) specific gene sequences from complex mixtures in a fashion analogous to that reported by Manning et al. (25). Although further studies are obviously required, our results indicate that enzymatically biotin-labeled polynucleotides can function as nucleic acid affinity reagents.

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